ANTIBODY ENGINEERING FOR EXPRESSION IN INSECT CELLS AND LARVAE

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ABSTRACT

Antibodies are currently deployed as the recognition component of sensors that detect biological threat agents. Previously, we developed an anti-botulinum toxin antibody using a bacterial host. However, antibodies expressed in bacteria lack molecular modifications made post-translationally by animal (eukaryotic) cells. It was therefore desirable to express Fab fragment antibody genes in insect cell lines and larvae. In this study, we improved an existing baculovirus expression vector by inserting the reporter gene DsRed, then modified and inserted the heavy and light chain genes encoding an anti-botulinum toxin-binding Fab antibody. The structures of all plasmids constructed were verified by restriction analysis and sequencing. Preliminary data demonstrate that DsRed and anti-botulinum Fab are both strongly expressed in larvae of *Trichoplusia ni*, suggesting that this system may be an economical manufacturing process for recombinant antibodies.

INTRODUCTION

Antibodies are the essential component in immunological sensors that detect biological warfare (BW) agents, conferring both sensitivity and selectivity for BW agents. The Army and the Joint Program Office for Bio-Defense (JPO-BD) purchase and field biosensor platforms (JBIDS, JPBDS, JBREWS, and PORTAL SHIELD systems) that incorporate antibodies produced in whole animals (polyclonal) and mammalian cell cultures (monoclonal) for BW agent detection.

Recombinant proteins can be expressed in nearly any organism into which the gene encoding the desired product can be inserted. However, the experience of many researchers has narrowed the suite of useful organisms to a handful that are easy to manipulate genetically, easy and economical to grow, and readily give up the desired product after gene expression. These include the bacterium *Escherichia coli*, the yeast *Pichia pastoris*, several mammalian cell lines, plants (e.g., tobacco and corn) and, most recently, intact whole organisms ranging from insects to goats and cattle. The production of recombinant proteins in insects poses the following challenge: a transfected insect looks like an unmodified insect. Therefore, determining the success of recombinant protein production requires either the extraction and analysis of the product from insects sampled from a production lot, or a means of detecting protein production without extraction.

The purpose of this study was two-fold. The first objective was to modify an existing insect cell expression vector to contain a gene that would allow external monitoring of the extent of gene expression in insect larvae. We chose the gene encoding DsRed, an auto-fluorescing protein derived from *Discosoma*

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coral. Fusions between DsRed and the gene encoding the desired protein would give rise to a protein whose amino-terminal end is the desired product, and whose carboxy -terminal end is DsRed. Accumulation of the desired product would therefore be tied directly to the accumulation of DsRed, which can be monitored optically. In this way larvae producing a desired recombinant protein can be easily identified and selectively harvested at a considerable savings over a process requiring direct assay for the desired product. The more red seen in the larvae, the greater quantity of product is produced. The second objective was to demonstrate the effectiveness of the expression of a useful protein product using the DsRed fusion vector. We chose to demonstrate the system using a gene set encoding an antibody that binds botulinum toxin ("BotFab"), inserting the two BotFab genes (heavy chain and light chain) into the DsRed fusion vector so that the antibody light chain gene and the DsRed reporter protein would be expressed as a single fusion protein. This fusion would allow the ready determination of the expression level of the antibody in a larva before beginning the process of antibody extraction and purification because the fluorescent protein and BotFab antibody would be produced simultaneously. This report describes the cloning of the anti-botulinum Fab heavy and light chain genes (BotFab 5) into the insect baculovirus expression vector pAcAB3, the modification of pAcAB3 to contain the DsRed gene, and the cloning of the heavy and light chain genes into the resulting pAcAB3-DsRed vector.

METHODS

BACTERIA, PLASMIDS, AND CULTURE CONDITIONS

The host strain for all cloning in this study was *Escherichia coli* DH5α[1], obtained from our in-house collection. Cultures of *E. coli* were grown at 37°C in LB broth[2] or on solid LB medium containing 1.5% agar. Carbenicillin was added to media for *E. coli* at 50 µg/ml. Plasmid pAcAB3 was purchased from BD Pharmingen (San Diego, CA). Plasmid pDsRed was purchased from BD Biosciences (formerly Clontech; Palo Alto, CA).

MOLECULAR BIOLOGY

Plasmid DNA was prepared for use as PCR template and for sequencing from cells of *E. coli* using QIAGEN maxi-columns (QIAGEN Inc., Valencia CA). Plasmids for cloning analysis were prepared from 2 ml cultures of *E. coli* with QIAGEN Spin Miniprep kits. PCR products were separated from unused and dimerized primers by gel electrophoresis[2], or purified with Qiaquick PCR Purification Kits. PCR products were extracted from agarose gel slices and purified using the Qiaquick Gel Extraction kit. All restriction enzymes were purchased from New England Biolabs (Beverly, MA) and used according to the manufacturer's instructions. PCR reactions contained 45 µl PCR Supermix (Life Technologies, Gaithersburg, MD), 1 µl (200 ng) of each primer, 1-3 µl of template DNA, and molecular biology-grade H_2O was added to a total volume of 50 µl.

DNA ligation reactions were set up as follows: 1 µl of T4 DNA ligase (NEB), 2 µl of T4 DNA ligase buffer, plus insert and vector DNA in varying amounts to bring the total volume of the reaction to 20 µl. Ligations were performed in thin-walled PCR tubes in a Perkin Elmer 9600 temperature cycler set to maintain a temperature of 16°C. Products of ligation were transformed into electrocompetent cells of *E. coli* strain DH5α and transformants were selected on solid LB medium containing carbenicillin.

Automated cycle sequencing reactions (fluorescent dye-terminator) were performed at the University of Maryland School of Medicine Biopolymer Laboratory, Baltimore, MD. DNA sequences were analyzed using LaserGene software (DNASTAR, Madison WI).

PCR PRIMER DESIGN

Primers were analyzed with Applera's Primer Express software, and secondary structure and dimer formation were checked with PrimerFinder software (http://eatworms.swmed.edu/~tim/primerfinder/). Primers were synthesized by The Midland Certified Reagent Co. (Midland, TX).

AMPLIFICATION AND CLONING OF DSRED

The gene encoding DsRed was amplified using primers 1 and 2. These primers were designed to amplify the DsRed open reading frame (ORF) and attach *Bgl*II and *Blp*I restriction sites on the 5'end and 3' end of the DsRed ORF, respectively. The forward PCR primer was also designed to include *Sac*I and *Avr*II restriction sites. These sites do not occur in plasmid pAcAB3, so their inclusion upstream of the DsRed ORF created two unique cloning sites into which genes of interest may be introduced to form translational fusions with DsRed. Plasmid pAcAB3 and the amplified DsRed fragment were then digested with *Bgl*II and *Blp*I, purified as described above, ligated as shown in Figure 1, and transformed into *E. coli.* DNA was isolated from transformants and cut with *Bgl*II and *Blp*I to verify the presence of DsRed insert DNA (Figure 1).

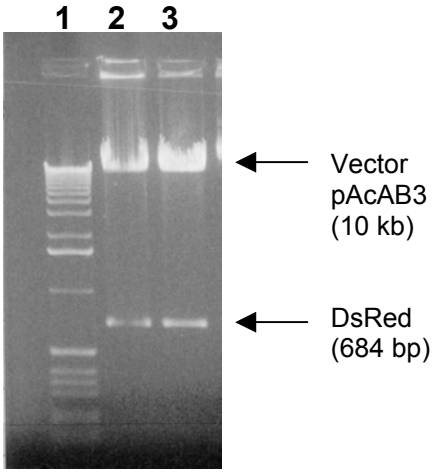

Figure 1. Verification of DsRed cloning. The plasmid resulting from the ligation of pAcAB3 and the amplified DsRed fragment was purified by miniprep, cut with *Bgl*II and *Blp*I, and analyzed by agarose gel electrophoresis. Two fragements of the expected sizes were generated, vector (10 kb) and insert (approximately 700 bp). Lane 1: 1 kilobase ladder size marker; lanes 2 and 3, digested pAcAB3-DsRed.

BOMBYXIN LEADER SEQUENCES

As constructed originally (Emanuel et al. 1996), the anti-botulinum antibody heavy and light chain genes were fused on the carboxyl-terminus to peptides derived from the *pelB* gene of *Xanthomonas campestris*. The *pelB* leader peptide targets the export of the heavy and light chain proteins from the cytoplasm of *E. coli* where they are translated from mRNA, to the periplasm where they fold and form the intact Fab antibody. The *pelB* leader peptides, however, do not have this function in insect cells, and therefore it was necessary to replace them with sequences that encode a peptide that would direct their export from the cells. The protein bombxyin is exported avidly from insect cells, and the leader peptide from this protein was chosen as the replacement for *pelB*. The replacement was performed as an integral part of PCR amplification of the heavy and light chain genes.

To perform the replacement, we synthesized PCR primers that had, at their 5' ends, the 3' portion of the bombyxin leader sequence, and the 5' end of the heavy (or light) chain at the 3' end (Figure 2, top PCR reactions). PCR primers for the distal ends of the heavy and light chain were designed to include restriction sites to facilitate their cloning into pAcAB3. When amplified from the bacterial expression vector, the antibody genes were preceded by bombxyin-encoding sequences.

ANTIBODY CLONING STRATEGIES

Two strategies for antibody expression in insect larvae were employed. In one, we amplified the bombyxin leader-light chain gene fusion with primers that added *Sac*I and *Avr*II restriction sites to allow the cloning of this gene into pAcAB3-DsRed, forming a bombxyin leader-light chain-DsRed fusion. The reverse PCR primers for this amplification also include a short sequence encoding an enterokinase cleavage site. This site is labile in the presence of enterokinase, a protease that will remove DsRed from the light chain after the fusion protein is produced and purified.

In the second strategy, we omitted the use of DsRed and cloned the bombxyin-light chain fusion directly into pAcAB3. This was done against the possibility that the fusion with DsRed might prevent proper assembly of heavy and light chains after expression. In both approaches, we amplified the heavy chain gene with a bombxyin leader sequence built into the forward amplification primers, and left intact the existing 6 x histidine tag at the 3' end of the heavy chain gene. This bombyxin leader-heavy chain gene-his tag fusion was cloned into the unique *Xma*I site in both pAcAB3-DsRed (Figure 2) and pAcAB3 (same as Figure 2 without DsRed).

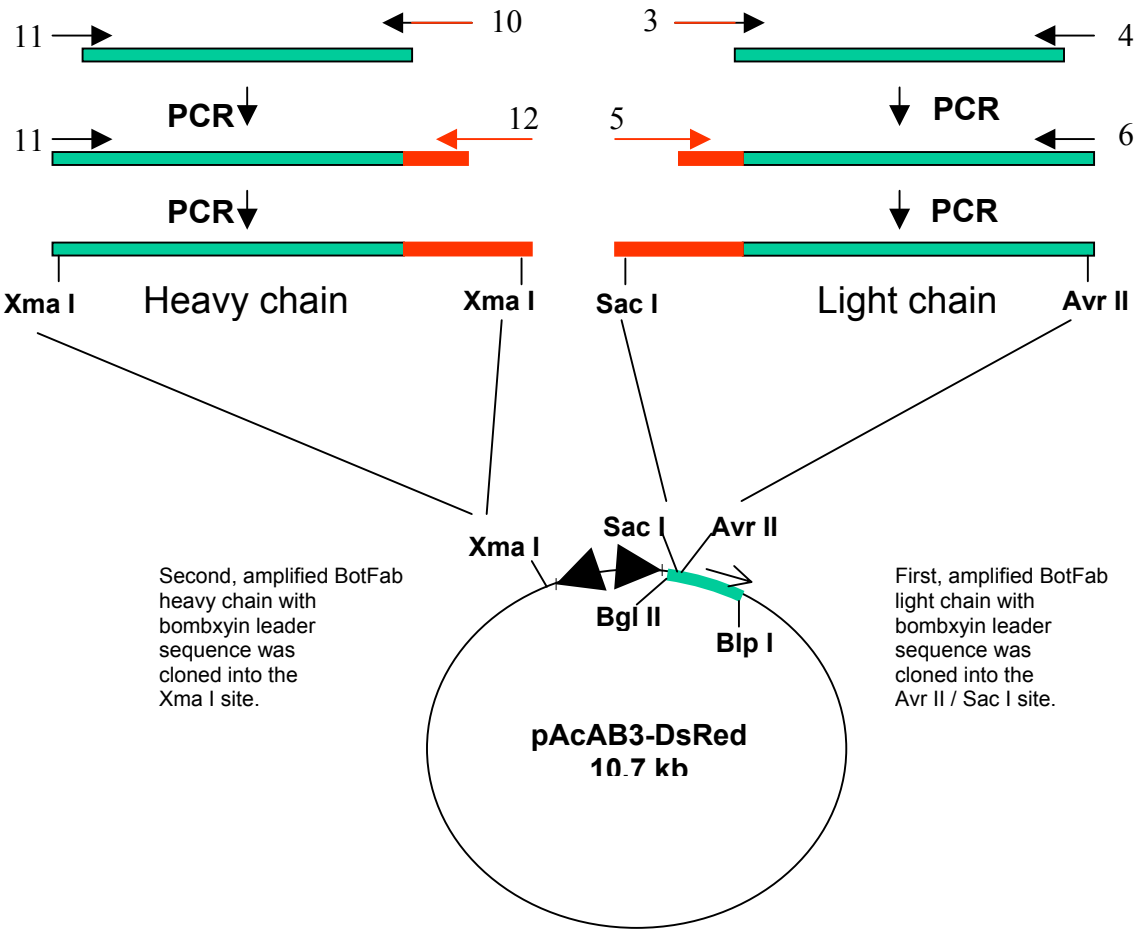

Figure 2. PCR amplification strategy for heavy and light chains and cloning strategy for vector pAcAB3-DsRed. Heavy and light chain sequences were amplified from plasmid pHist5[3] using forward primers

beginning with bombyxin leader sequence and ending with heavy or light chain sequence (top PCR reactions). Numbers correspond to primers used. A second amplification of both light and heavy chain genes was needed to complete the addition of bombyxin sequence and add the 5' cloning sites to each gene (second PCR reaction). Light chain was cloned in first by necessity, as heavy chain is cut by SacI. Divergent baculovirus p10 promoters (large black triangles) drive expression of heavy and light chain genes in insect cells after transfection.

VERIFICATION OF CLONING RESULTS

The insertion of light chain and heavy chain into each of the two vectors was confirmed by a set of restriction digests, with the desired result being predicted by analysis of the final sequences as assembled by computer. Figure 3 shows the *Sac*I / *Avr*II digest of pAcAB3-DsRed containing the light chain gene. Additional digests were performed to verify that the light chain was inserted as a single copy (data not shown). Similar experiments were performed to verify the insertion of light chain into vector pAcAB3 (data not shown).

HEAVY CHAIN AMPLIFICATION AND CLONING

Heavy chain sequences were amplified in the order shown in Figures 3 and 4, using pHist5 as template. The resulting heavy chain gene fused to the bombyxin leader peptide was digested with *Xma*I, and several attempts were made to clone this fragment into both pAcAB3 (light chain) and pAcAB3-DsRed (light chain). All of these attempts were unsuccessful, due to the possibility that *Xma*I might cut the PCR product poorly, resulting in low cloning efficiencies.

To circumvent this, we first cloned the final heavy chain PCR product into the unique *Xma*I site of cloning vector pUC18. This was done because the efficiency of cloning fragments into smaller vectors is much higher than when cloning into vectors 10 kb and larger. The resulting plasmid, pUC18-heavy chain, was then digested with *Xma*I, and the heavy chain separated from pUC18 by gel electrophoresis (Figure 4). This operation ensured that all heavy chain fragments recovered were in fact cleaved with *Xma*I at both ends before attempting to insert them into either of the larger vectors containing the light chain gene.

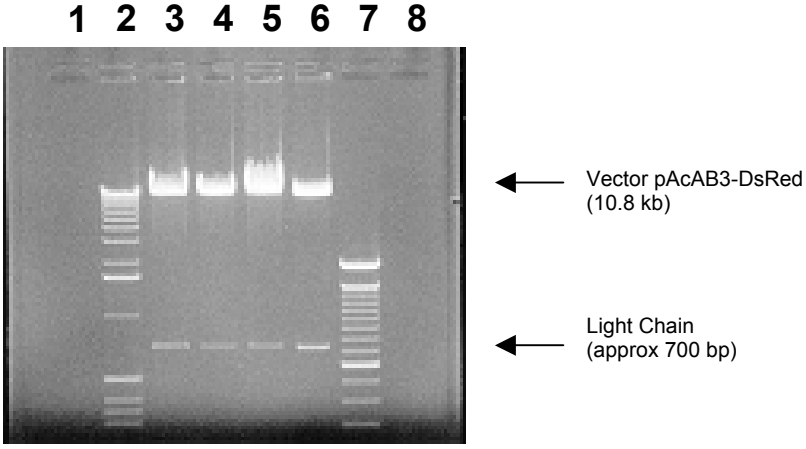

Figure 3. Verification of the cloning of light chain into vector pAcAB3-DsRed. Lanes 1 and 8, no sample. Lane 2, 1 kilobase ladder (size marker). Lanes 3-6, miniprep DNA from four of the colonies obtained after cloning, digested with *Sac*I and *Avr*II, showing successful cloning attempts. Lane 7, 100 base-pair ladder.

The heavy chain gene was cloned into each vector only after light chains were inserted. This was necessary because the heavy chain sequence contains restriction sites for *Blp*I (which would have interfered with light chain cloning if performed second in pAcAB3-DsRed) and *Sac*I (which would have interfered with light chain cloning if performed second in pAcAB3). The heavy chain could not be cloned "directionally", as only one suitable unique restriction site in pAcAB3 is located downstream of the p10 promoter divergent from that driving light chain expression. Therefore, the single *Xma*I site had to be used, and the orientation and copy number of the heavy chain determined only after cloning (data not shown). Restriction digests that were instructive were: *Xma*I, *Bgl*II + *Spe*I, *Bam*HI, *Stu*I, and *Blp*I.

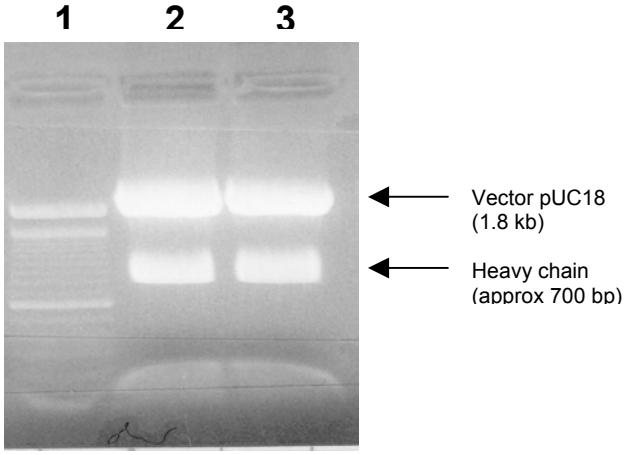

Figure 4. Cloning of heavy chain into, and digestion from, vector pUC18. Lane 1, 100 bp ladder. Lanes 2 and 3, *Xma*I digest of pUC18-heavy chain construct. Heavy chains were excised from the gel, purified, and ligated to *Xma*I-digested pAcAB3-DsRed (light chain) or pAcAB3 (light chain).

RESULTS

The structures of the plasmids constructed in this study were verified by direct sequencing (U. of MD School of Medicine core facility). Introduction of the DsRed construct into baculovirus and subsequent infection of *T. ni* larvae has demonstrated very striking results: larvae expressing quantities of DsRed that are so great that they turn bright pink, observable with the unaided eye (data not shown). DsRed expression was also noted to be strong in cultured Sf9 cells (an insect cell line) (data not shown).

Chesapeake PERL staff also infected both cells and larvae with the vector containing the antibody genes without DsRed. Preliminary western results have shown that both the heavy and light chains of the Botfab antibody, cloned without the DsRed fusion, are well expressed (Figure 5). Some larvae were estimated to produce as much as 1-3 mg/larva. Samples of the antibody were purified and tested with control lots of the Botfab to compare the affinities and specific activities (Figure 6).

A complete demonstration of the system will include the expression of a desired recombinant protein in larvae fused to DsRed, the identification of high producers visually using DsRed as the marker, the purification of the recombinant protein, and determination that the recombinant protein has the desired activity. Features designed to test all these aspects of the system have been engineered into the vectors, as described in this report.

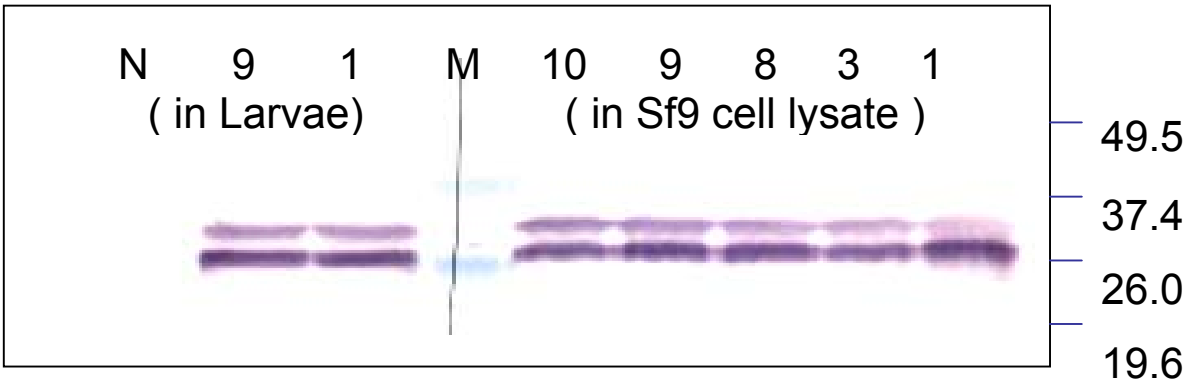

Figure 5. Western blot analysis of proteins produced from vAcAB3/light chain/heavy chain in *T. ni* larvae (worms 1 and 9) and Sf9 cell lysate (tubes 10, 9, 8, 3, and 1). N represents the proteins produced by the normal worm and M is the molecular weight marker.

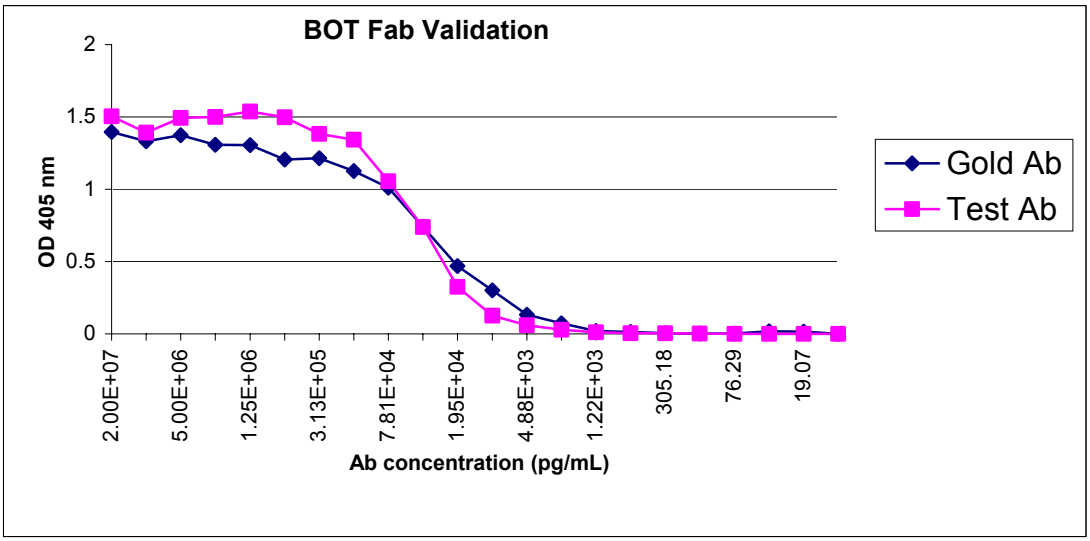

Figure 6. Comparison of ELISA performed using the BOT Fab extracted from the bacteria *E. coli* as the "Gold Ab" and the BOT Fab extracted from the insect cell line Sf9 as the "Test Ab". The results indicate a similar level of sensitivity between the antibodies.

CONCLUSIONS

Variations of each feature of the system are possible, giving future investigators the ability to engineer around problems that may arise that are particular to individual products. For example, DsRed fusions to a recombinant protein might provide inconsistent red marking of larvae because the fusion partner perturbs the folding of DsRed. In such a case, DsRed could still be used as an indicator of the extent of viral infection in a larva by expressing it from another strong promoter in the same vector. For example, the vector used in this study, pAcAB3, has three suitable promoters, of which two were used. Similarly, if the assembly of a multi-polypeptide protein proves inefficient, the system could still be

useful for the expression of single polypeptide proteins. This simplicity and adaptability are two of the most powerful benefits of the larval expression system.

Gene expression in insect cells should provide a useful means for the production of materials for human therapeutics. The manufacture of products for use in humans requires a strict adherence to cGMP (current Good Manufacturing Practices) to ensure the safety, quality and purity of product. The system described here should be amenable to cGMP production when insect cells are used as the production system. Cell cultures, just like larvae, can be monitored spectrophotometrically for the extent of protein expression, and the point at which protein expression is optimal can be numerically defined. Under cGMP, a similar quantification of product will likely need to be defined in a larva-based production regime. Also, since all facets of a manufacturing process must be controlled under cGMP, a production line relying on transfected larvae will need to obtain a supply of larvae or eggs that is similarly controlled and maintained.

Lastly, an economic analysis will need to be performed once data on product yield is obtained. Preliminary data indicate that, in the case of Botfab, approximately 300-1000 larvae may be required to produce one gram of antibody. Other proteins will need to be expressed, and the cost of raising, infecting, growing/harvesting larvae, and subsequent purification of product will have to be determined. Since several technological hurdles have already been overcome for the automation of a larval production line, it seems likely that only minor molecular modifications will be required to obtain an efficient production line. Market analysis, including the going rates for recombinant proteins made by other methods, and the overall demand for recombinant proteins, will ultimately determine the economic viability of this insect protein production system.

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